

Cellular basis of urothelial squamous metaplasia: roles of lineage heterogeneity and cell replacement

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Although the epithelial lining of much of the mammalian urinary tract is known simply as the urothelium, this epithelium can be divided into at least three lineages of renal pelvis/ureter, bladder/trigone, and proximal urethra based on their embryonic origin, uroplakin content, keratin expression pattern, *in vitro* growth potential, and propensity to keratinize during vitamin A deficiency. Moreover, these cells remain phenotypically distinct even after they have been serially passaged under identical culture conditions, thus ruling out local mesenchymal influence as the sole cause of

their *in vivo* differences. During vitamin A deficiency, mouse urothelium form multiple keratinized foci in proximal urethra probably originating from scattered K14-positive basal cells, and the keratinized epithelium expands horizontally to replace the surrounding normal urothelium. These data suggest that the urothelium consists of multiple cell lineages, that trigone urothelium is closely related to the urothelium covering the rest of the bladder, and that lineage heterogeneity coupled with cell migration/replacement form the cellular basis for urothelial squamous metaplasia.

Introduction

Metaplasia occurs when one differentiated cell type is replaced by another, usually as an adaptive response to chronic irritation. Because metaplastic changes frequently precede neoplasia, they are sometimes regarded as precancerous lesions (Leube and Rustad, 1991). Almost all epithelial tissues are known to be able to undergo metaplasia. For example, respiratory epithelium of the nasal cavity and bronchial/bronchiolar mucosa can undergo squamous metaplasia in response to tobacco smoke, inflammation, viral infection, and certain carcinogens (Leube and Rustad, 1991). The stratified squamous epithelium of the lower esophagus can transform into an intestinal epithelium-like structure complete with goblet cells as a result of gastroesophageal reflux ("Barrett's esophagus"; Peters et al., 2004). Despite the potential importance of metaplasia in some disease processes, the cellular basis of metaplasia is frequently a subject of debate.

It is well known that vitamin A deficiency can induce keratinizing squamous metaplasia in a variety of epithelia (Wolbach and Howe, 1925; Molloy and Laskin, 1988). However, this keratinizing transformation, as occurring in esophageal,

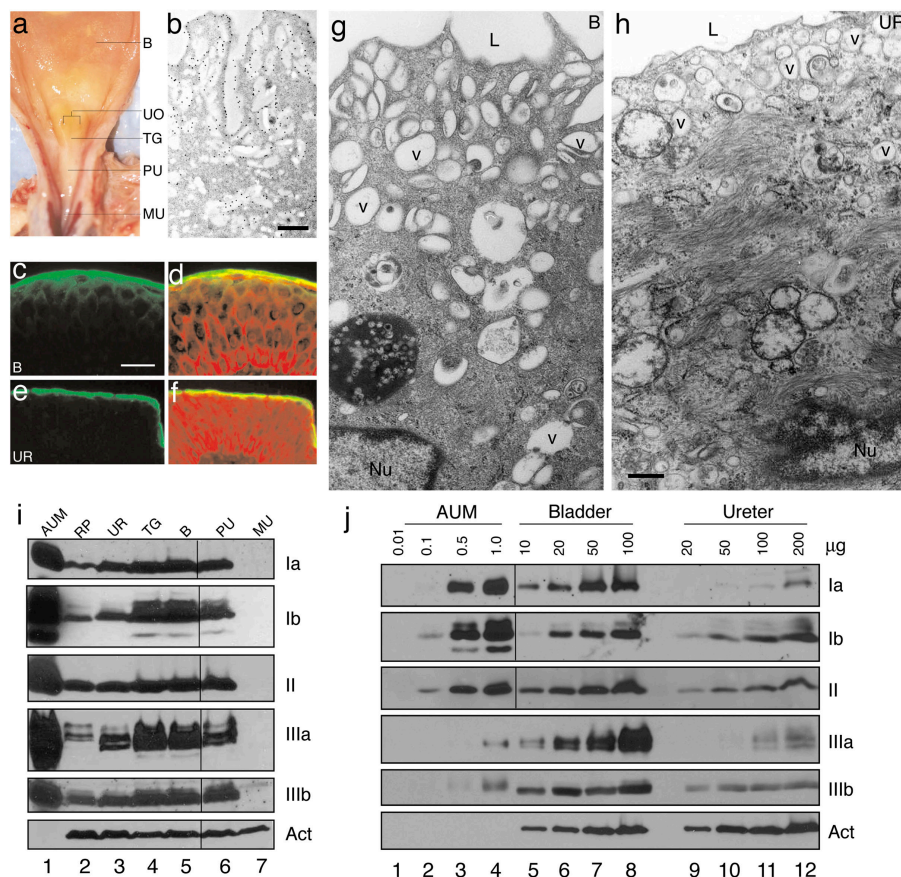
corneal, and conjunctival epithelia, is quite incomplete (Tseng et al., 1984). In contrast, some seemingly random areas of rat and mouse bladder urothelium can, in response to vitamin A deficiency, undergo complete keratinization (orthokeratinization), forming an epithelium that is morphologically hardly distinguishable from the epidermis (Wolbach and Howe, 1925; Molloy and Laskin, 1988). It is puzzling, however, that bladder urothelial keratinization occurs heterogeneously, with fully keratinized areas adjacent to areas lined with apparently normal urothelium (Hicks, 1968; Gijbels et al., 1992). The cellular basis and biological significance of such a strikingly heterogeneous metaplasia of bladder urothelium has not been explained.

The urothelium, also known as transitional epithelium, lines much of the urinary tract, including the outer medulla portion of the renal pelvis, ureters, bladder, and proximal urethra (Hicks, 1968; Sun et al., 1999; Lewis, 2000; Apodaca, 2004; Staack et al., 2005). It forms an extraordinarily effective permeability barrier, and it can withstand a lifetime of repeated stretch and contraction (Hicks, 1968; Sun et al., 1999; Lewis, 2000; Apodaca, 2004). To perform these functions, the apical surface of urothelium becomes highly specialized, as it is covered almost completely by 16-nm uroplakin (UP) particles that are packed hexagonally, forming two-dimensional crys-

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Abbreviations used in this paper: AUM, asymmetric unit membrane; PCNA, proliferative cell nuclear antigen; UP, uroplakin.

Figure 1. Bovine urothelium is morphologically and biochemically heterogeneous. (a) The lower bovine urinary tract around the trigone area (male). B, bladder; MU, membranous urethra; PU, prostatic urethra; UO, ureteral orifices; TG, trigone. (b) Electron microscopy immunolocalization of UP IIIa in bovine urothelial umbrella cells. (c–f) Double immunofluorescent staining of the bovine bladder (c and d) and ureter (UR; e and f) using antibodies to UP IIIa (c and e; monoclonal antibody AU1; green) and keratins (d and f; a rabbit antiserum against total keratins; red; double exposure) showing more uniform cytoplasmic staining of bladder urothelial umbrella cells than the ureteral cells. (g and h) Transmission electron microscopy of the umbrella cells of bovine bladder (g) and ureteral (h) urothelium showing more abundant cytoplasmic UP-delivering vesicles (V) in bladder urothelial cells than ureteral cells. L, lumen; Nu, nucleus. (i) Immunoblot analysis of the total proteins of bovine urothelia that were isolated by scraping from the renal pelvis (RP), ureter, trigone, bladder, prostatic urethra, and membranous urethra using antibodies to UPs Ia, Ib, II, IIIa, and IIIb. Actin (Act) and the total UPs of bovine urothelial plaques (AUM) were used as a loading control and reference, respectively. (j) Semiquantification of UPs. Various amounts of total proteins (as indicated) from purified bovine AUM (lanes 1–4), scraped bovine bladder urothelium (5–8), and ureteral urothelium (9–12) were immunoblotted using antibodies to individual UPs as indicated. The immunoblot experiments were performed twice with similar results. Note that UPs in 10–20 μg of bladder urothelium were equivalent to those present in 100–200 μg of ureteral cells, suggesting that bladder epithelium contained ~ 10 times more UPs than ureteral urothelium on a per total cellular protein basis. Bars (b), 1 μm ; (c–f) 50 μm ; (g and h) 1 μm .



tals (called urothelial plaques or asymmetric unit membrane [AUM]). Four major (Ia, Ib, II, and IIIa) and one minor (IIIb) UP have been identified so far in purified bovine urothelial plaques (Wu and Sun, 1993; Lin et al., 1994; Wu et al., 1994; Yu et al., 1994; Deng et al., 2002). These UPs are synthesized as major urothelial differentiation products, and they form specific heterodimers (UPIa/UII and UPIb/UIIIa or IIIb) before they can exit the endoplasmic reticulum (Deng et al., 2002; Tu et al., 2002; Hu et al., 2005). Ablation of UP genes compromises urothelial permeability barrier function (Hu et al., 2000, 2002). Although urothelia of the ureter and trigone region of the bladder are thought to be mesoderm derived, whereas those of the rest of the bladder and prostatic urethra are endoderm derived (Baker and Gomez, 1998), urothelia of these various regions are morphologically similar, and it has been presumed that they perform similar functions (Hicks, 1965).

In this paper, we show that the urothelia of the various regions of the mammalian urinary tract can be divided into at least three different cell lineages (renal pelvis/ureter; bladder, including the trigone area; and proximal urethra) and that during vitamin A deficiency, some of the urethral urothelial basal cells form keratinized foci that expand horizontally to replace the surrounding normal urothelium. These results demonstrate the cellular basis of urothelial squamous metaplasia and provide an explanation for the remarkable heterogeneity of bladder keratinization.

Results

Urothelia of the bovine bladder and ureter are ultrastructurally and biochemically distinct

Bovine tissues are particularly suitable for analyses of possible urothelial heterogeneity because (1) we can readily isolate sufficient amounts of relatively pure urothelial cells from various parts of the urinary tract for UP analyses (Fig. 1 a); (2) we have generated antibodies that are monospecific to all bovine UPs (Wu et al., 1994; Liang et al., 2001); and (3) we can serially cultivate bovine urothelial cells (Surya et al., 1990; Sun et al., 1999). As shown previously, UP antibodies strongly labeled not only the apical surface-associated urothelial plaques (AUM) but also the cytoplasmic UP-delivering vesicles (Fig. 1 b; Wu et al., 1990; Wu and Sun, 1993; Liang et al., 2001). Immunofluorescent staining showed that although UPs were associated with the apical surface of both bladder (Fig. 1, c and d) and ureteral urothelia (Fig. 1, e and f), the cytoplasm of the bladder umbrella cells stained more uniformly and strongly than that of the ureter. This result was consistent with the electron microscopic data showing that the bladder umbrella cells had many more cytoplasmic UP-delivering vesicles (Fig. 1 g) than the corresponding ureteral cells (Fig. 1 h). To confirm that the bladder urothelial cells contained more UPs than ureteral cells, we analyzed the total proteins of these two epithelia as

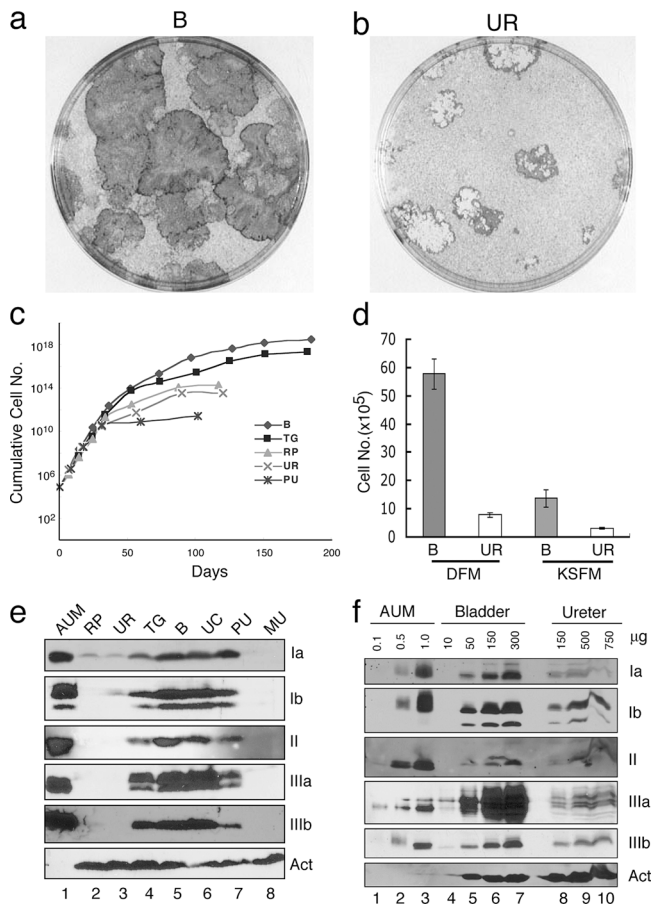


Figure 2. Bovine bladder and ureteral urothelial cells remain morphologically and biochemically distinct when cultured under identical in vitro conditions. Bovine bladder urothelial cells (a) and ureteral urothelial cells (b) form morphologically distinct colonies in the presence of 3T3 feeder cells (300 urothelial cells plated per 60-mm dish containing 5×10^5 mitomycin-treated 3T3 feeder cells). (c) Bovine bladder (B) and trigone (TG) urothelial cells have a much higher in vitro growth potential, as measured by cumulative cell number and by a later onset of senescence than the urothelial cells of the renal pelvis (RP), ureter (UR), and proximal urethra (PU). Similar results were obtained from two independent experiments. (d) The in vitro growth advantage of the bladder over ureteral urothelial cells could be seen in both DFM (a 1:1 mixture of DME and F-12 medium containing 1.05 mM calcium) or in KFSM (keratinocyte serum-free medium containing 0.09 mM calcium; Surya et al., 1990). 10^5 urothelial cells were plated in DFM medium in the presence of 3T3 feeder cells (5×10^5 per 60-mm dish) or in KFSM medium in the absence of the feeder, and the urothelial cells were trypsinized and counted after 6 d. The results shown are averages of quadruplicates \pm SD (error bars). (e) Immunoblot analyses of the UPs (using antibodies with indicated specificities) and actin (Act) synthesized by cultured bovine urothelial cells of the renal pelvis, ureter, trigone, bladder, urachus (UC), prostatic urethra (PU), and membranous urethra (MU; $\sim 150 \mu\text{g}$ of total cellular proteins each). UPs of AUM ($5 \mu\text{g}$) were used as controls. (f) Semicantification of the UPs synthesized by cultured urothelial cells of bovine bladder and ureter showing that cultured bladder urothelial cells contained ~ 10 times more UPs than cultured ureteral urothelial cells. The immunoblot experiments were performed twice with similar results.

well as the bovine renal pelvis, trigone, prostatic urethra, and membranous urethra (of males) by immunoblotting. With the exception of the membranous urethra, which was UP negative, all of the urothelia were found to contain UPs Ia, Ib, II, IIIa, and IIIb (Fig. 1 i). The relative sizes of UPs of the various urothelia were identical to those of the bladder except renal

pelvis, whose UP IIIa was slightly larger than that of the bladder (Fig. 1 i), possibly reflecting a different degree of glycosylation (Hu et al., 2005). The results indicated that ureteral and renal pelvis urothelia indeed contained less UPs than the bladder (including the trigone) and urethral urothelia (Fig. 1 i). To study this more carefully, we compared the immunoblot intensities of various urothelial UPs with known amounts of bovine AUM UPs (Chen et al., 2003). The results indicated that 10–20 μg of total bladder urothelial proteins contained about the same amount of UPs as in 100–200 μg of total ureteral urothelial proteins (Fig. 1 j), indicating that the UP content of the bladder urothelium was ~ 10 times higher than that of the ureteral epithelium.

The phenotypic differences of various bovine urothelia persisted in culture

To determine whether different local stromal environments were responsible for the observed in vivo phenotypic differences among the various urothelia (Cunha et al., 1983; Li et al., 2000), we compared the growth and differentiation properties of bovine bladder and ureteral urothelial cells that were serially cultured under identical in vitro conditions. In the presence of 3T3 feeder cells, bladder cells formed colonies that were stained much more intensely by Rhodanille blue than ureteral cells as a result of increased stratification (Fig. 2, a and b; and not depicted). Moreover, urothelial cells of the bladder wall and the trigone had a higher in vitro proliferative potential than those of the renal pelvis, ureteral, and proximal urethra, as measured by their cumulative cell numbers (Fig. 2 c). Similarly, monkey bladder urothelial cells grew better than ureteral urothelial cells (not depicted). The superior in vitro growth potential of bovine bladder urothelial cells could be demonstrated not only in our standard urothelial growth medium containing 1.05 mM calcium (with feeder cells) but also in a serum-free medium (Surya et al., 1990) containing 0.09 mM calcium (without feeder; Fig. 2 d). Finally, immunoblot analyses showed that the UP contents of cultured bovine bladder wall, trigone, and urethral urothelial cells were ~ 10 times higher than those of cultured renal pelvis and ureteral urothelial cells (Fig. 2, e and f; and Table I).

Heterogeneous responses of mouse bladder urothelia to vitamin A deficiency

To analyze the metaplastic responses of various urothelial compartments, we fed mice with a vitamin A-deficient diet (see Materials and methods). Mouse bladders and associated structures were serially sectioned to visualize proximal urethra, trigone, bladder wall, and bladder dome, and sections were stained with antibodies to keratin K10 (marker for keratinization; Fig. 3, a, c, e, and g) and to UPs (Fig. 3, b, d, f, and h). The K10 and UP staining, as seen in adjacent sections, were found to be mutually exclusive. Keratinization of the urothelia occurred much earlier (~ 8 wk of age) in female than in male mice (~ 48 wk). Studies of female mice that had been on vitamin A-deficient diets for various lengths of time (8–56 wk) showed that keratinizing squamous metaplasia occurred initially in the proximal urethra and trigone (Fig. 3, a–d) and expanded upwards (Fig. 3, e–h), although the very top of the

Table I. The *in vivo* and *in vitro* properties of various types of bovine urothelia

Location ¹	Embryonic origin	<i>In vivo</i> ²				<i>In vitro</i> ³			Lineage ⁴
		Cell layers	UPs	K14	K1/K10	Cell layers	UPs	Growth potential	
Renal pelvis	Mesoderm	+ / ++	+	–	–	+	+	+	R/U
Ureter	Mesoderm	+++	+	–	–	+	+	+	R/U
Trigone	Endoderm	+++	+++	–	–	++	++	+++	B/T
Bladder	Endoderm	+++	+++	–	–	++	++	+++	B/T
Proximal urethra	Endoderm	+++	+++	+ / –	+ / –	++	++	+	PU
Distal urethra	Ectoderm	+++	–	+++	+++	ND	ND	ND	–

¹Trigone refers to the area defined by ureteral orifices and the opening of the bladder neck; proximal urethra begins at the top of bladder neck and ends when the urothelium becomes UP negative (in the male, this zone includes the bladder neck and prostatic urethra).

²Based on mouse and bovine data.

³Based on bovine data.

⁴R/U, renal pelvis/ureter; B/T, bladder/trigone; PU, proximal urethral.

dome was rarely involved (Table II; schematically summarized in Fig. 3 m). The K10- and UP-positive epithelia were morphologically indistinguishable from the epidermis and normal urothelium, respectively, and these two epithelia always maintained a sharp boundary (Fig. 3, i–l), with no intermediate cells expressing both K10 and UP markers (Fig. 3, a–h).

To better understand the cellular basis of urothelial heterogeneity, we stained sections of various zones of the normal female mouse urinary tract using antibodies to UPs and various keratins (Fig. 4). Control experiments showed that, as expected, the anti-K14 and -K1 antibodies stained mouse epidermis and vaginal epithelium mainly basally and suprabasally, respectively (Fig. 4, a and b), and that the anti-K10 antibody recognized a single 56.5-kD (K10) keratin in the total protein extract of the mouse skin epidermis (Fig. 4 c, lane 1) and proximal urethra (Fig. 4 c, lane 2), thus clearly establishing the specificities of the antibodies. Consistent with our previous results (Lin et al., 1994; Zhang et al., 1999), all suprabasal cell layers of the bladder dome urothelium expressed UPIIIa (Fig. 4 d) with no detectable K14 (a basal keratinocyte marker; Fig. 4 e) or K1 and K10 (keratinization markers; Fig. 4, f and g; Woodcock-Mitchell et al., 1982; Sun et al., 1984). The urothelium of the proximal urethra was also UP positive, although the staining was more limited to the superficial cell layer (Fig. 4 h). Some of its basal cells that were scattered expressed K14 (Fig. 4, i–k), and, unexpectedly, most of its basal and intermediate cells were K1 and K10 positive (Fig. 4, l and m). In the male mouse, the prostatic urethral epithelium exhibited UP/keratin patterns (Fig. 4, n and o) similar to those of the female proximal urethra. As expected, the epithelial lining of the spongy (of males) and distal urethra (of females) did not express UP but rather expressed K1/K10 suprabasally (Fig. 4, p and q), which is consistent with their close relation to the ectoderm-derived epidermis. Similar results were obtained with bovine urothelia.

To identify the target cells that were responsible for the formation of keratinized squamous epithelium during vitamin A deficiency, we stained serial sections of the mouse urinary tract using antibodies to K14 (marker for keratinocyte basal cells), K1 (marker for keratinization), and UPs (markers for urothelial differentiation). We found that as early as 8 wk after the female mice had been fed with a vitamin A–deficient diet, keratinizing epithelial foci that were K14 positive (basal), K1

positive (suprabasal), and UPIIIa negative were formed (Fig. 5, a–h, asterisks). The squamous nature of these epithelia was confirmed by the fact that proliferative cell nuclear antigen (PCNA)–positive cells were restricted to the basal layer (Fig. 5, i, j, and l) unlike the urothelium, where such cells could be associated with all cell layers, including the superficial umbrella cells (Fig. 5, i–k; Kong et al., 2004).

Discussion

At least three urothelial lineages: renal pelvis/ureter, bladder/trigone, and proximal urethra

Based on their ultrastructure and UP contents, bovine urothelia can be divided into at least three types (Table I). Those of the renal pelvis and ureters have lower UP contents than those of the bladder wall, the trigone zone of the bladder, and the prostatic (proximal) urethra (Fig. 1, i and j). To determine whether the observed phenotypic differences are caused by different local mesenchymal signals (extrinsic modulation), we compared the phenotypes of bladder and ureteral cells that had been serially cultured under an identical *in vitro* environment. If the two cell types remain phenotypically distinct even after they have extensively replicated in culture (thus diluting out their *in vivo* mesenchymal cues), they must have diverged from each other during development and represent two separate cell lineages (intrinsic divergence). We have previously used this approach to establish that keratinocytes of the skin, cornea, and esophagus represent distinct cell lineages and to illustrate the importance of environmental influences in modulating epithelial differentiation (Doran et al., 1980). In this study, we have established that bovine urothelial cells of the bladder and ureters, when maintained under identical *in vitro* conditions, form morphologically distinct colonies (Fig. 2, a and b), that they have very different *in vitro* proliferative potentials (Fig. 2, c and d), and that they contain vastly different amounts of UPs (Fig. 2, e and f). These results are consistent with previous findings that the endoderm-derived bladder urothelium and the mesoderm-derived ureteral urothelium respond differently to the same embryonic seminal vesicle mesenchymal instruction to adopt the prostatic and seminal vesicle phenotype, respectively

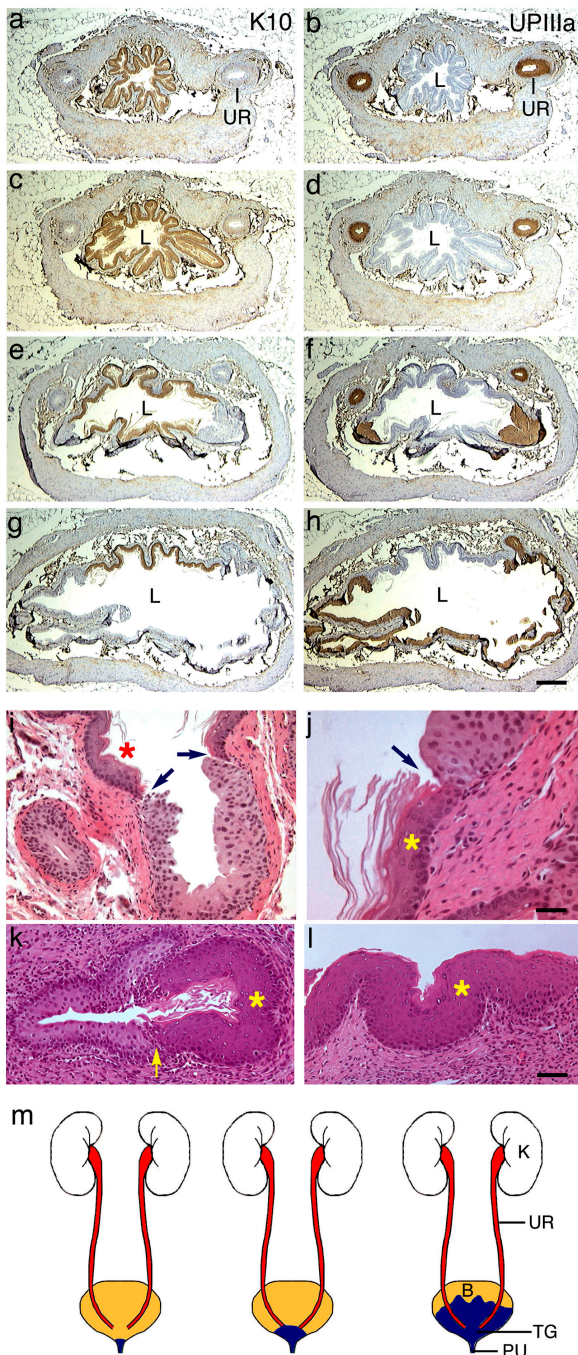


Figure 3. Focal urothelial keratinization in the proximal urethra and the maintenance of a sharp boundary between the advancing keratinized epithelium and the retreating normal urothelium. The urinary tract of vitamin A-deficient mice (28-wk-old female; see Materials and methods) was dissected, and adjacent sections were stained with either a monoclonal antibody to keratin K10 (a, c, e, and g) or with the AU1 antibody to UP IIIa (b, d, f, and h). Sections shown in a and b are from the trigone, whereas those in g and h are from the bladder wall; sections shown in c-f are from intermediate zones. Note the complete keratinization of the trigone urothelium (K10 positive and UP negative) and the largely normal urothelium (K10 negative and UP positive) of the bladder wall. (i-l) Hematoxylin- and eosin-stained sections showing the sharp boundary (arrows) between the keratinized epithelium (asterisks) and the normal-appearing urothelium. (m) Depicts schematically the expansion of the K10-positive epithelium, which originates from the proximal urethra and the trigone area and expands to the rest of the bladder except the top dome. B, bladder; K, kidney; L, lumen; UR, ureter; PU, proximal urethra; TG, trigone. Bars (a-h), 400 μ m; (i) 100 μ m; (j, k, and l) 200 μ m.

Table II. The heterogeneous responses of mouse urothelium to vitamin A deficiency

Age (wk)	Male		Female	
	Dome	Neck	Dome	Neck
8	-	-	-	+
12	-	-	-	+++
16	-	-	-	++++
24	-	-	-	++++
28	-	-	-	++++
44	-	-	-	++++
48	-	+	ND	ND
52	-	+++	ND	ND
56	-	++++	-	++++

Normal urothelium, + to +++++, denotes increasing degrees of keratinization. -, nonkeratinized.

(Cunha, et al., 1991; Donjacour and Cunha, 1993). Altogether, these data strongly suggest that urothelia of the bladder and ureter represent two separate cell lineages that are most likely maintained by distinct stem cell populations.

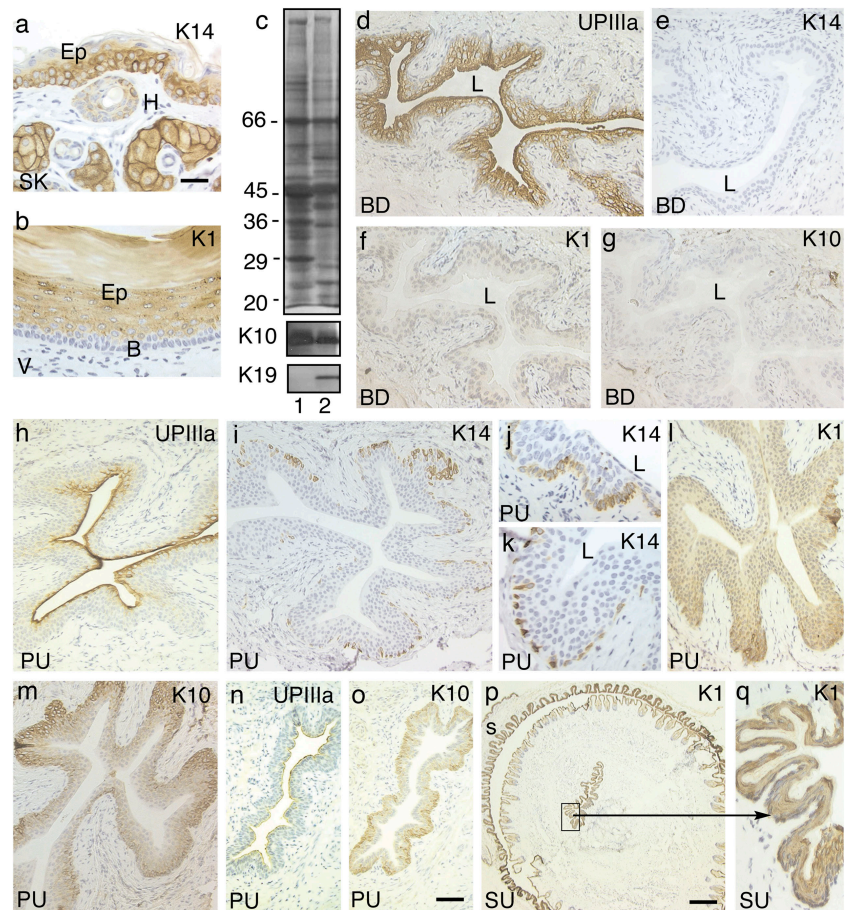
Our data also indicate that cultured urothelial cells of the renal pelvis and ureter have indistinguishable in vitro growth and differentiation properties and, thus, probably belong to the same lineage (Fig. 2, c and e); different local mesenchymal signals may account for the observed morphological variations between these two urothelia. Although it has been suggested that trigone and bladder urothelia are endoderm and mesoderm derived, respectively, the in vitro growth and differentiation properties of these two cell types are indistinguishable, suggesting that they may belong to the same lineage (Fig. 2, c and e; see below). Finally, studies on the in vitro proliferative potential suggest that urothelial cells of the prostatic (proximal) urethra are distinct from those of the bladder and trigone and, thus, belong to a third lineage (Fig. 2 c and Table I).

Although most of our data were obtained using bovine and mouse urothelial cells, our preliminary data indicate that monkey bladder urothelial cells also grew better than ureteral urothelial cells in culture. Similarly, under the same culture conditions, human embryonic ureteral and bladder urothelial cells remain distinguishable, although the former consistently grew better than the latter under our culture conditions (unpublished data). Together, these findings indicate that the concept of urothelial heterogeneity is applicable not only in the bovine and mouse but also in the monkey and human.

Mechanisms and significance of urothelial metaplasia

Our finding that vitamin A deficiency in mice initially causes keratinizing squamous metaplasia of urothelial cells of the proximal urethra, which normally synthesizes small amounts of K1 and K10 keratins, is interesting in several respects. The prostatic (proximal) urethral urothelium expresses these two keratins in their basal and intermediate cell layers (Fig. 4, l, m, and o) instead of in the suprabasal cells as in keratinized epidermis or vaginal epithelium (Fig. 4 b). A similar basal expression occurs with the differentiation-dependent K3 keratin, which is expressed suprabasally in cultured corneal epithelial

Figure 4. Expression of UPs and keratins in the urothelia of the mouse urinary tract. Paraffin sections of mouse skin (SK; a) and vaginal epithelium (V; b), bladder dome (BD; d–g), proximal urethra (PU; h–m for female and n and o for male), and spongy urethra (SU; p and q) were stained immunohistochemically using various antibodies to detect UP IIIa and keratins K1, K10, and K14 as indicated. (c) SDS-PAGE and immunoblot analyses of the total proteins of mouse skin (lane 1) and proximal urethral urothelium (lane 2) by silver nitrate staining (top) and immunoblotting using anti-K10 antibody (middle) and anti-K19 antibody (bottom). Note that the bladder dome urothelium was UP IIIa positive and K1/K10 negative and, in contrast, that the urothelium of the proximal urethra was UP positive in its superficial cell layer, K14 positive in some of its basal cells, and K1/K10 positive in its basal and intermediate cells. The boxed area in panel p is shown at a higher magnification in panel q. Ep, epidermis; H, hair follicle; L, lumen; B, bladder; S, skin. Bars (a, b, j, k, and q), 100 μ m; (d–i and l–o) 200 μ m; (p) 400 μ m.



cells and in the limbal epithelium (the corneal epithelial stem cell zone); however, K3 is expressed in all cell layers (basal included) of central corneal epithelium (the nonstem cell zone; Schermer et al., 1986). The basal expression of K1/K10 in the bulk of urethral urothelium raises the interesting possibility that these regions correspond to the central corneal epithelium and suggests that a search for the K1/K10-negative urothelial basal cells may lead to the identification of certain urothelial stem cells (see next paragraph). In this study, these K1/K10 keratins provide useful markers for tracing the migration of the proximal urethra–originated urothelial cells during squamous metaplasia (Fig. 3) and for the identification of small keratinizing foci during early stages of urothelial keratinization (Fig. 5).

There are several possible mechanisms (which are schematically illustrated in Fig. 6) that can potentially explain the striking heterogeneity in urothelial metaplasia and the sharp boundary between the keratinized epithelium and the seemingly normal urothelium. Because we have not found any intermediate cells expressing both urothelial and keratinization markers, we can largely rule out the direct transformation of umbrella cells (Fig. 6 A) or the dedifferentiation of umbrella cells followed by redifferentiation (Fig. 6 B). Given our current finding that there are several distinct urothelial lineages likely to be maintained by separate stem cells, our data are inconsistent with the idea that the entire urinary tract is populated by a single population of pluripotent urothelial stem cells that give rise to different normal urothelia as well as to the keratinized

epithelium during vitamin A deficiency (Fig. 6 C). On the other hand, we cannot rule out the possibility that within the proximal urethral urothelium, there exist two separate populations of basal (stem) cells. Because we observed multiple small foci of keratinization in urethral urothelium (Fig. 5), our results are consistent with the presence of some (K14 positive) basal cells within the urethral urothelium that are scattered and intermingled with normal (K14 negative) urothelial basal cells (Fig. 4, i–k); these K14-positive cells are activated during vitamin A deficiency to give rise to a keratinized epithelium (Fig. 6 D). We speculate that the K14-positive basal cells are responsible for the formation of the keratinized foci because only a small number of basal cells are K14 positive in normal urethral urothelium, similar to the keratinized foci. The fact that most of the basal cells are K1/K10 positive practically rules out the possibility that all of these K1/K10-positive cells are stem cells. This situation is analogous to the corneal/limbal epithelium, in which the limbal stem cells are K14 positive and K3/K12 (equivalent to K1/K10 of the keratinized epithelia) negative, whereas the central corneal epithelial basal cells (that are probably transit-amplifying cells) are K14 negative and K3/K12 positive (Schermer et al., 1986; Cotsarelis et al., 1989). Therefore, it is possible that proximal urethral urothelium contains a subpopulation of K14-positive (and K1/K10 negative) basal cells that are selectively activated during vitamin A deficiency, forming keratinized foci (Fig. 6 D) that later fuse and expand. In addition, our results on vitamin A–deficient mice

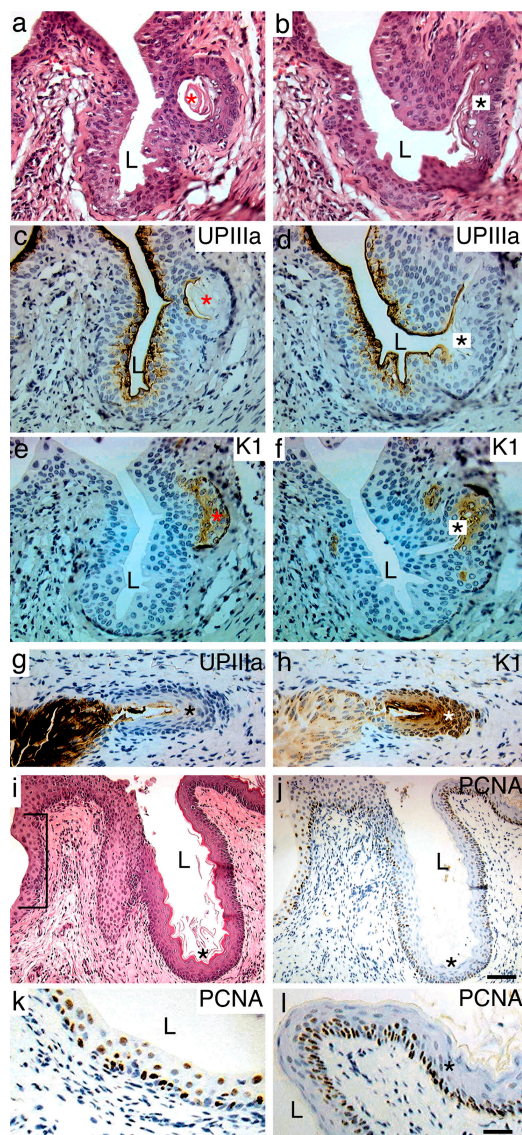


Figure 5. The initial formation of keratinized foci in the mouse bladder urothelium during vitamin A deficiency. Serial sections of the urinary tracts of female mice that had been kept on vitamin A-deficient diets for 8–56 wk (a–f, 8 wk; g and h, 12 wk; and i–l, 16 wk) were stained with hematoxylin and eosin (a, b, and i), AU1 mouse monoclonal antibody to UP IIIa (c, d, and g), a rabbit antiserum to keratin K1 (e, f, and h), and a mouse monoclonal antibody to PCNA (j–l). Panels a, c, and e; b, d, and f; g and h; and i and j were serial sections. Bracket in panel i indicates an area of normal urothelium. Note the formation of small keratinized foci that contained K1-positive and UP-negative suprabasal cells (asterisks in a–h). Also note that the PCNA-positive cells were present in all cell layers in the nonkeratinized epithelium (k) but were mainly restricted to the basal layer in keratinized epithelium (l). L, lumen; asterisks denote keratinized regions in all panels. Bars (i and j), 200 μ m; (a–h, k, and l) 100 μ m.

suggest that keratinizing squamous metaplasia originates from the urothelium of the proximal urethra and trigone area and that this keratinized epithelium expands into other parts of the bladder (Figs. 6 E, 3 m, and Table II). This idea is supported by the finding that the proximal urethra–originated keratinized epithelium seems to always maintain a sharp boundary with the normal-looking “retreating” urothelium (Fig. 3). A related finding was made by Varley et al. (2004), who described a sharp

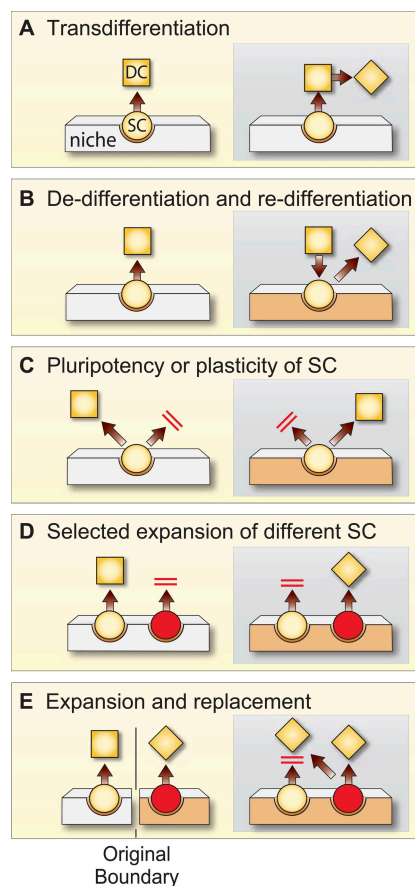


Figure 6. Models of epithelial metaplasia. (A) A terminally differentiated cell (DC; square) can, in response to environmental changes (gray), directly transform into a different type of terminally differentiated cell (diamond; the transdifferentiation model). (B) The terminally differentiated cells can convert back to a relatively undifferentiated (or stem) cell, which can then, in response to environmental and/or mesenchymal changes (beige), differentiate along a different pathway, yielding a distinct phenotype (the dedifferentiation and re-differentiation model). (C) Under normal conditions, the pluripotent stem cells give rise to terminal differentiated cells of a particular phenotype; environmental changes may induce such stem cells to undergo an alternative pathway of differentiation. The recently demonstrated plasticity of certain stem cells, in response to strong mesenchymal niche influence, may fit this model (pluripotent or plastic stem cell model). (D) The tissue contains two separate populations of stem cells: one of them normally gives rise to the (dominant population of) terminally differentiated cell types, whereas the other lays dormant. Environmental changes, including the alteration of the stem cell niche, suppress the growth and differentiation of the originally dominant stem cell (yellow) while activating the originally dormant stem cell (red) that now gives rise to an entirely different phenotype (the selective expansion model). The activation of the esophageal gland stem cell in Barrett’s esophagus (Gillen et al., 1988; Kumagai et al., 2003), the vitamin A deficiency–induced squamous metaplasia of tracheal epithelium (Wolbach and Howe, 1925; Nasiell, 1963), uterine epithelium (Ponnamperuma et al., 1999), and cervical epithelium (Darwiche et al., 1993) may fall into this category. (E) The tissue contains two separate cell lineages that occupy different domains separated with well-defined boundaries. Environmental changes such as vitamin A deficiency favor the expansion of one cell lineage over another, thus allowing one cell type to expand and invade into another cell lineage’s domain (the cell lineage heterogeneity and replacement model). This last model can best explain our data on urothelial keratinizing squamous metaplasia that is induced by vitamin A deficiency. (C–E) The parallel red bars denote that the process/pathway is blocked. SC, stem cell.

boundary between normal-appearing transitional epithelium (K20+, K14–, and K13 basal/intermediate) and squamous epithelium in the human bladder (K20–, K14+, and K13 suprabasal).

Overall, our data can best be explained by urothelial heterogeneity in combination with the model in Fig. 6 E, although the model in Fig. 6 D may operate in the initial formation of keratinized foci in the proximal urethral urothelium.

It has been suggested that squamous metaplasia is a precursor of bladder cancer (Coulson, 1989; Akdas and Turkeri, 1991). For example, bilharzial infection causes bladder wall inflammation, leading to urothelial squamous metaplasia and squamous cell carcinoma formation. Although urothelium is normally extremely slow, cycling with a labeling index of $\sim 0.1\%$ (Jost, 1986), urothelium undergoing squamous metaplasia becomes hyperplastic, with a greatly elevated labeling index of $>4\%$ (Fig. 5, k and l; Hicks, 1968). Given the fact that hyperplasia itself is a risk factor for bladder cancer formation (Sakata et al., 1988; Cohen and Ellwein, 1990; Su et al., 2003), it is possible that the hyperplasia component of urothelial squamous metaplasia is a major contributor of an enhanced risk of bladder cancer formation.

Biological implications of urothelial heterogeneity

This refined understanding of urothelial lineage has several implications: (1) The term urothelium should be used in conjunction with a description of its tissue origin (e.g., bladder urothelium, ureteral urothelium, etc.). Using the term without specifying its origin can be confusing and potentially misleading. (2) It is known that the renal pelvis and ureter, which we found to have similar properties, are mesoderm derived, whereas the bladder and urethra are endoderm derived (Baker and Gomez, 1998). Whether the trigone urothelium is mesoderm or endoderm derived has been controversial (Gosling and Dixon, 1995; Batourina et al., 2002; Foster et al., 2004). Our data, as shown in Fig. 2 (c and e), strongly support the idea that the urothelium of the trigone, like that of the rest of the bladder, is endoderm instead of mesoderm derived (Batourina et al., 2005; Thomas et al., 2005). (3) Although the mammalian bladder has a somewhat higher frequency of tumor formation than the ureters and renal pelvis, this has usually been attributed simply to a larger bladder surface area and to a possibly longer exposure time of the bladder urothelium to urinary carcinogens. It is interesting to note, however, that deficiency in essential fatty acids can cause papillary transitional cell tumors mainly in the renal pelvis and upper portion of the ureter (Monis and Eynard, 1980). Moreover, Nortier et al. (2000) reported that urothelial carcinoma caused by the use of a weight-reducing Chinese herbal medicine that was contaminated by aristolochic acid, a potent carcinogen, occurred almost exclusively in the renal pelvis and ureter. These data clearly establish that different urothelia can respond to the same carcinogens differently. (4) Functionally, the urothelium of the bladder may be subjected to a more extensive and longer stretch than that of the ureter. This may explain why, in comparison with ureteral urothelium, bladder epithelium accumulates many more cytoplasmic UP vesicles that can fuse with the apical surface to increase the surface area, thus preventing cell rupture during bladder distention (Staehelin et al., 1972). (5) Because human ureters are more readily available from

surgical specimens of kidney donors and nephrectomies than the bladder and because human ureteral urothelial cells are easier to culture than bladder cells (unpublished data), one may be tempted to use ureteral urothelial cells as a convenient substitute for bladder urothelium in various basic studies (Hicks, 1965) as well as in clinical applications such as bladder reconstruction and augmentation (Li et al., 2000; Atala, 2002; Mitchell, 2003; Southgate et al., 2003; Staack et al., 2005). However, given the finding that urothelia are heterogeneous, one needs to keep in mind that these two urothelia may have different growth and differentiation properties, functional performance, and tumor-forming capacities. (6) It is well known that the trigone area of the normal human female bladder tends to undergo squamous metaplasia with histological features that are somewhat similar to vaginal epithelium (vaginal metaplasia; Long and Shepherd, 1983; Shirai et al., 1987). Stephenson et al. (1989) reported that the basal and suprabasal cells of such metaplastic epithelial cells express nuclear estrogen receptor and, thus, are distinct from the neighboring normal trigonal urothelial cells. Although these results are said to support the notion that trigone epithelium is embryologically distinct from the urothelial lining of the rest of the bladder, we believe that this reflects the propensity of the proximal urethra-derived urothelial cells to invade the trigone region—a process frequently associated with chronic inflammation (Ito et al., 1981; Long and Shepherd, 1983).

Materials and methods

Nomenclature

In both males and females, the urothelium covers the renal pelvis, ureters, trigone, and the rest of the bladder. In males, the trigonal urothelium is contiguous with the UP-positive prostatic urethra followed by the UP-negative membranous and spongy urethra. In females, the trigonal urothelium is contiguous with the UP-positive proximal urethral urothelium followed by the UP-negative distal urethral epithelium. We consider the UP-positive prostatic urethra of males to be equivalent to the UP-positive proximal urethra of females and, for simplicity, sometimes call them both proximal urethra (Romih et al., 2005).

Tissues and immunohistochemical staining

Fresh bovine (male) and mouse (C57Bl/6xDBA/2-F1; also known as B6D2F1 or BDF; both sexes) tissues were obtained from a local slaughterhouse and from Taconic, respectively. All of the laboratory animal protocols were approved by the Institutional Animal Care and Use Committee of the New York University School of Medicine. Tissue specimens were immediately fixed in 10% formalin in PBS, paraffin embedded, and sectioned at 4 μm . Paraffin sections were stained with hematoxylin and eosin or were stained immunohistochemically using antibodies against UPs (mouse AU1 monoclonal antibody to UP11a [Liang et al., 2001] or rabbit antisera to various UPs), keratins (the mouse monoclonal AE1 and AE3 antibodies; Woodcock-Mitchell et al., 1982; Eichner et al., 1984), keratin 10 (Chemicon International, Inc.), keratin 13 (AE8; Pang et al., 1993), or keratins 1, 5, and 14 (Babco). Samples were visualized with a microscope (Axiophot; Carl Zeiss Microimaging, Inc.) with plan-Apochromat 10/0.32 and 20/0.60 or plan-Neofluar 40/0.75 objective lenses. Images were captured with a digital camera (DKC-5000; Sony) at room temperature. Electron microscopy was performed on a microscope (JEM 200 CX; JEOL; Kodak Electron Microscope film 4489) as described previously (Liang et al., 2001). The images were processed in size and contrast/brightness with Adobe Photoshop 6.0.

Vitamin A-deficient mice

Pregnant female mice (B6D2F1; Taconic) were fed with a vitamin A-deficient diet (TD-88407; Harlan Teklad) beginning on day 12 of pregnancy (Molloy and Laskin, 1988; De Luca et al., 1989). Both the lactating

mother and the litters were maintained on the same diet for 3 wk, after which the mice were separated by sex and kept on the same diet for up to 56 wk of age. The control mice were fed the same diet from birth, to which vitamin A had been added at 19,824 IU/kg of diet (TD-88406). After the mice were killed by CO₂ narcosis and cervical dislocation, the kidneys, ureters, bladders, and urethras were removed en bloc and fixed in 10% formalin (in PBS); the bladder was also gently inflated with the fixative.

Polyacrylamide gel electrophoresis and immunoblotting

Total urothelial proteins from urothelia that were isolated by scraping were resolved by SDS-PAGE on 17% polyacrylamide gel, transferred electrophoretically to nitrocellulose, and incubated with primary antibodies and with secondary peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies (Liang et al., 2001).

Cell culture

Tissues from different regions of the urinary tract were washed with PBS and treated with Dispase (2.5% wt/vol in DME; GIBCO BRL) at 37°C for 1 h. The detached, intact urothelial sheets were isolated and dissociated by incubating in 0.25% trypsin (in PBS) for 20 min at 37°C. The cell suspensions were filtered through three layers of sterile gauze, collected by centrifugation, and plated in DFM medium (a 1:1 mixture of DME and F-12 culture media; GIBCO BRL; 4×10^5 per 60-mm dish) in the presence of mitomycin-treated 3T3 feeder cells (5×10^5 per 60-mm dish; Rheinwald and Green, 1975; Surya et al., 1990). The cumulative cell number was determined by subculturing and plating urothelial cells at 5×10^4 per 60-mm dish in the presence of 3T3 feeders and by counting the cells when they reached ~80% confluence. In some experiments, urothelial cells (10^5) were plated in the keratinocyte serum-free medium (GIBCO BRL). The colony-forming efficiency was determined by plating 300 cells in the presence of 3T3 feeder cells followed by fixing and staining the dish with Rhodanille blue 14 d later (Rheinwald and Green, 1975).

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